Express Mail No. EM 061 024 310 US

CONTROL OF GENE EXPRESSION IN EUKARYOTES

1. INTRODUCTION

This invention relates to an inducible gene expression system, particularly but not exclusively eukaryotes, such as plants, for example.

2. BACKGROUND TO THE INVENTION

Manipulation of plants to improve certain characteristics requires the control of expression of foreign or endogenous genes in plant tissues. Such manipulation relies on the availability of mechanisms to control gene expression as required. It is therefore advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be used. A range of promoters is known to be operative within plants.

Within the promoter there are several defined domains which are necessary for the function of the promoter. The first of these domains is located immediately upstream of the structural gene and forms the core promoter region, about 70 base pairs immediately upstream of the genes. This region contains the CAAT and TATA boxes and represents a transcription initiation sequence which defines the transcription start site for the gene. A series of regulatory sequences upstream of the core promoter sequence constitute the remainder of the promoter. The regulatory sequences determine the expression levels, the spatial and temporal pattern of expression and possible expression under inductive conditions.

The control of expression of heterologous genes in plant cells is important for the successful genetic manipulation of plants to alter and/or improve phenotypic characteristics. Promoters and/or regulatory sequences from bacteria, viruses, fungi and plants have been used to control gene expression in plants. In some cases it will be desirable to control the time and/or extent of the expression of introduced genetic material in plants, plant cells or tissue. The ability to regulate the expression of transgenes provides several important advantages: (1) regulation of expression of gene(s) that might interfere with the transformation and regeneration process (Roeder *et al.*, 1994, McKenzie *et al.*, 1998), (2) reversible control of gene expression at a specific time (e.g. manipulation of carbon metabolism by Caddick *et al.*, 1998 and secondary product formation by Sommer *et al.*, 1998), (3) control of growth and development (e.g. flowering, plant fertility, cell wall formation), (4) control of genes that respond to environmental signals (e.g. attack by pathogens, such as, for example, nematodes, arachnids or aphids), (5) expression of

selectable marker genes and (6) expression of recombinase proteins at specific time points. Each of these applications can use the inducible gene expression system and novel sequences of the present invention.

2. 1 Known regulatable gene expression systems in plants

A few plant genes are known to be induced by a variety of internal and external factors including plant hormones, heat/cold shock, chemicals, pathogens, lack of oxygen and light. Few of these systems have been described in detail.

Ideally a chemically inducible activating promoter in a 5' regulatory region should have low background activity in the absence of an inducer and demonstrate high expression in the presence of an inducer. A chemically inducible repressing promoter in a 5' regulatory region should have low background activity in the presence of an inducer and demonstrate high expression in the absence of an inducer. The activator/repressor should also only allow control of the transgene. This renders the use of most endogenous promoters unsuitable and favors the use of those better characterized regulatory elements of model organisms distant in evolution, such as yeast, *E. coli*, *Drosophila* or mammalian cells, that respond to signals that are usually not encountered in higher plants. These characteristic regulatory elements are, however, less advantageous in their operation than the system proposed in the present invention.

On this basis, two different concepts of gene control can be realized, namely promoter-repressing systems and promoter-activating systems.

2.2 Promoter-repressing systems

The repression principle is based on the sterical interference of a protein with the proteins important for transcription. It is a common mechanism in bacteria, for example LexA, Lac and Tet, but occurs much less frequently in higher eukaryotes. Two bacterial repressor/operator systems (Lac and Tet) have been used to control the activity of promoters transcribed by RNA polymerase II. Gatz and Quail (1988) taught the use of the Tn10-encoded Tet repressor/operator with a cauliflower mosaic virus 35S promoter in a transient plant expression system. Frohberg *et al.*, (1991) and Gatz *et al.*, (1991, 1992) characterised the effect of placing Tet operator sequences at different positions in a CaMV 35S promoter. US Patent No. 5,723,765 and International Patent Application, Publication No. WO 96/04393 disclosed use of the Tet repressor system for the inducible expression of the Cre recombinase in transgenic plants. Wilde *et al.*, (1992) used the Lac repressor/operator system for the inducible expression from a chlorophyll a/b binding protein promoter in protoplasts of stably transformed plants.

2.3 Promoter-activating systems

A second approach for the construction of a chemically inducible system is to use transcriptional activators from higher eukaryotes. The mammalian glucocorticoid receptor (GR), which activates eukaryotic expression only in the presence of steroids has been used by Picard *et al.*, (1988) in *Schizosaccharomyces pombe*. Schena *et al.*, (1991) have shown that transcription from a target promoter containing GR-binding sites was strictly dependent on the addition of steroids in transiently transformed tobacco cells. Lloyd *et al.*, (1994) have used a fusion of the steroid receptor protein with the maize transcription factor R to complement an *Arabidopsis* mutant in a steroid inducible fashion. Aoyama and Chua (1997) disclosed use of a chimeric transcription factor consisting of the DNA-binding domain of the yeast transcription factor Gal4, the transactivating domain of the herpes viral protein Vp16 and the receptor domain of the rat glucocorticoid receptor to induce the expression of a reporter gene in transgenic plants through the application of steroids.

International Patent Application, Publication No. WO 96/27673 describes the use of a steroid receptor system in transgenic plants using chimeric GR receptors with Vp16 and C1 transcriptional activation domains and Gal4 DNA binding domains.

Another eukaryotic ligand-dependent activator is Ace1, a copper-dependent transcriptional activator from yeast. Mett *et al.*, (1993) have shown that Ace1 regulates the expression of a suitable target promoter (CaMV 35S –90 bp promoter containing the Ace1 binding site) in transgenic plants. McKenzie *et al.*, (1998) used a similar system (Ace1 binding sites with a CaMV 35S –40 bp promoter) to investigate copper-inducible activation of the *ipt* gene in transgenic tobacco.

AlcR is the specific activator of the *Aspergillus nidulans* ethanol-utilisation pathway, mediating the induction of its own transcription and that of the structural genes *alcA* and *aldA*. AlcR is a DNA binding protein that recognises specific binding sites in structural gene promoters (Kulmburg *et al.*, 1992, Fillinger & Felenbok 1996). Felenbok (1991) used the *AlcA-AlcR* system for the expression of recombinant proteins in *Aspergilli*. The ethanol inducible gene switch was used by Caddick *et al.*, (1998) to manipulate carbon metabolism in transgenic plants and also by Salter *et al* (1998) to examine the induction of a chloramphenicol acetyltransferase (CAT) reporter construct by ethanol. This system has also been used in International Patent Application, Publication No. WO 93/21334 for the inducible activation of a chimeric *alcA*/CaMV 35S promoter in transgenic plants.

2.4 Fusion proteins

A third strategy is based on the construction of fusion proteins between transcriptional transactivation domains and bacterial repressor proteins such as the Lac and the Tet repressor. Weinmann *et al* (1994) used a tetracycline controlled transactivator (the virus protein 16 (Vp 16) activation domain fused to the Tet repressor protein) to switch off expression of a GUS transgene in transgenic plants in the presence of the inducer.

2.5 Mutant Repressor Proteins

A fourth strategy is based on the creation of mutant repressor proteins that bind to DNA only in the presence of the inducer. Gossen *et al.*, (1995) have developed a reverse Tet repressor protein that binds to DNA only in the presence of the inducer and used this system successfully in mammalian cells.

Very recently, in International Patent Application No. PCT/GB98/01893 work was carried out at *Rhodococcus* sp. V49 in respect of biosensor materials and methods of uses thereof. *Rhodococcus* sp. V49 (formerly *Nocardia corallina*) ATCC19070 is a non-acid fast, gram-positive rod-shaped soil bacterium. It can use a range of monoaromatic compounds, including 3-(2-hydroxyphenyl)propionic acid (orthohydroxyphenylpropionic acid, OHP) and 2-hydroxy cinnamic acid as the sole carbon source. It is also able to grow on n-hexadecane, benzene and toluene. The international patent application, the subject matter of which is to be deemed incorporated herein, discloses the nucleotide sequence of the 7.5 kb OHP operon from *Rhodococcus* sp. V49.

The polypeptide encoded by the ohpR gene shows a strong sequence similarity throughout its length to a number of bacterial transcriptional regulators from the GntR family (Haydon & Guest 1991). The strong sequence similarity indicates that ohpR encodes a prokaryotic transcriptional regulator.

International Patent Application No. PCT/GB98/01893 discloses the use of genetically manipulated mycolic acid bacteria cells solely as sensors for analytes in environmental samples. The potential other uses and modifications of the novel nucleotide sequences described in the present invention are nowhere contemplated in PCT/GB98/01893.

3. SUMMARY OF THE INVENTION

The present invention provides a method of controlling eukaryotic gene expression comprising transforming a eukaryotic cell with an inducible gene expression system, the gene expression system comprising a first nucleotide sequence comprising a 5' regulatory region operably linked to a nucleic acid sequence which encodes a regulator polypeptide and an untranslated 3' termination sequence, and a second nucleotide sequence

comprising a 5' regulatory region operably linked to a nucleic acid sequence which is a coding or non-coding sequence, the expression of the nucleic acid sequence of the second nucleotide sequence being controlled by the regulator polypeptide of the first nucleotide sequence using an inducer, the inducer thereby causing modulation of expression of the nucleic acid sequence of the second nucleotide sequence, and the nucleotide sequence of the regulator polypeptide and/or the 5' regulatory region, or parts thereof, of the second nucleotide sequence being isolated from a prokaryote source.

The present invention also provides a chimeric gene comprising a first nucleotide sequence comprising a 5' regulatory region operably linked to a nucleic acid sequence which encodes a regulator polypeptide and an untranslated 3' termination sequence, and a second nucleotide sequence comprising a 5' regulatory region operably linked to a nucleic acid sequence which is a coding or non-coding sequence, the expression of the nucleic acid sequence of the second nucleotide sequence being controlled by the regulator polypeptide of the first nucleotide sequence using an inducer, the inducer thereby causing modulation of expression of the nucleic acid sequence of the second nucleotide sequence, and the nucleotide sequence of the regulator polypeptide and/or the 5' regulatory region or parts thereof of the second nucleotide sequence being isolated from a prokaryote source.

Advantageously, the regulator polypeptide comprises one or more domains, which domains may be a ligand binding domain, a nucleic acid binding domain, a transactivation domain, a targeting domain, a silencing/repressing domain or a dimerization domain. The regulator sequence may thus comprise a chimeric gene of different sequences.

3.1 DEFINITIONS

In order to provide a clear and consistent understanding of the specification and terms used herein, the following definitions are provided:

3.1.1 Regulatable Gene

A gene containing at least one regulatable nucleic acid sequence and at least one associated coding or non-coding nucleic acid sequence. The genes may be of natural, synthetic or partially natural/partially synthetic origin.

3.1.2 Inducer

An elemental or molecular species which controls, for example, initiates, terminates, increases or reduces, by direct or indirect action, the activity of a regulatable nucleic acid sequence in a system in which the inducer is not normally found in an active

form in an amount sufficient to effect regulation of transcription, to the degree and at the time desired, of transcribable nucleic acid sequence associated with the regulatable nucleic acid sequence.

This terminology embraces situations in which no or very little inducer is present at the time transcription is desired or in which some inducer is present but increased or decreased regulation is required to effect more or less transcription as desired. Thus, if the system containing the regulatable nucleic acid sequence is, for example, a transgenic plant, an inducer is a species not naturally found in the plant in an amount sufficient to effect regulation/modulation, and thus transcription of an associated gene, to the desired degree at the time desired.

By "direct action" it is intended that the inducer action results from the direct interaction between the inducer and the nucleic acid sequence. By "indirect action" it is meant that the inducer action results from the direct interaction between the inducer and some other endogenous or exogenous component in the system, the ultimate results of that direct interaction being activation or suppression of the activity of the nucleic acid sequence. By "active form" it is intended that the inducer be in a form required to effect control.

3.1.3 Regulator Polypeptide

This term as used herein refers to polypeptides which modulate the expression of a target gene (the nucleic acid sequence of the second nucleotide sequence of the present invention) in response to an inducer. The regulator polypeptide may comprise one or more of a ligand binding domain, a nucleic acid binding domain, a transactivation domain, a targeting domain, a silencing/repressing domain or a dimerization domain.

3.1.4 Chimeric Sequence or Gene

A nucleic acid sequence containing at least two parts, e.g. parts derived from naturally occurring nucleic acid sequences which are not associated in their naturally occurring states, or containing at least one part that is of synthetic origin and not found in nature.

3.1.5 Coding sequence

A nucleic acid sequence which, when transcribed and translated, results in the formation of a polypeptide.

3.1.6 Non-coding Sequence

A nucleic acid sequence which is not transcribed and translated, resulting in the formation of a polypeptide when associated with a particular coding nucleic acid sequence. Thus, for example, a sequence that is non-coding when associated with one coding sequence may actually be coding when associated with another coding or non-coding sequence.

3.1.7 Plant Tissue

Any tissue of a plant in planta or in culture. This term includes, but is not limited to, whole plants, plant cells, plant organs, plant seeds, protoplasts, callus, cell culture and any groups of plant cells organized into structural and/or functional units. The use of this term in conjunction with, or in the absence of, any specific type of plant tissue as listed above or otherwise embraced by this definition is not intended to be exclusive of any other type of plant tissue.

3.1.8 Modulation

The increasing or decreasing of the level of expression of a gene or the level of transcription of a nucleic acid sequence. The definition is not intended to embrace any particular mechanism.

4. DESCRIPTION OF THE FIGURES

In order that the invention may be easily understood and readily carried into effect, reference will now be made, by way of example, to the following diagrammatic drawings, wherein:

Figure 1 shows a schematic diagram of the plasmid pSK-489 as used in the present invention. The plasmid contains the nucleotide sequence for ohpR (from nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) inserted between the EcoRI and NotI sites in pBluescript;

Figure 2 shows a schematic diagram of the plasmid p35SC1 (Tuerck & Fromm 1994) as used in the present invention. The plasmid contains the C1 cDNA as described in Paz-Ares *et al.*, (1987) inserted as an EcoRI fragment between a CaMV 35S promoter, Adh1 intron 1 and a CaMV 35S terminator;

Figure 3 shows a schematic diagram of the plasmid pSK-483 as used in the present invention. The plasmid contains the C1 coding region as described in Paz-Ares *et al.*, (1987) inserted between the EcoRI and the NotI sites in pBluescript;

Figure 4 shows a schematic diagram of the plasmid pSK-59 as used in the present invention. The plasmid contains part of the ohp operator (nucleotide 1036 to nucleotide 1449 of SEQ ID NO: 1) inserted between the XhoI and SalI sites in pBluescript;

Figure 5 shows a schematic diagram of the plasmid pSK52040 as used in the present invention. The plasmid contains the CaMV 35S promoter, a GUS intron (Vancanneyt *et al.*, 1990) and a CaMV 35S terminator in pBluescript;

Figure 6 shows a schematic diagram of the plasmid pSK58040 as used in the present invention. The plasmid contains the ohp operator from nucleotide 1036 to nucleotide 1449 of SEQ ID NO: 1 inserted in plasmid pSK52040 into the XhoI site upstream of the CaMV 35S –90 bp core promoter. Downstream of the CaMV 35S core promoter are located a GUS intron and a nos terminator;

Figure 7 shows a schematic diagram of plasmid pDV35S1 as used in the present invention. The plasmid contains the CaMV 35S promoter and the CaMV 35S terminator in pBluescript;

Figure 8 shows a schematic diagram of plasmid pDV60 as used in the present invention. The plasmid contains the chimeric promoter of SEQ ID NO: 19, and the CaMV 35S terminator in pBluescript. The chimeric promoter in Seq. ID. 19 contains a 36 bp region of the ohp operon (from nucleotide 1225 to nucleotide 1260 of SEQ ID NO: 1) inserted into the CaMV35S promoter at nucleotide – 21;

Figure 9 shows a schematic diagram of plasmid pSK60040 as used in the present invention. The plasmid contains the chimeric promoter described in Figure 8 above (Seq. ID. 19), a GUS intron (Vancanneyt *et al.*, 1990) and a nos terminator in pBluescript;

Figure 10 shows a schematic diagram of plasmid pSK-490 as used in the present invention. The plasmid contains the chimeric regulator being a translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the NarI at nucleotide 536 to the end of the coding region at nucleotide 839, amino acids 179 to 279 of the C1 protein) inserted into pBluescript between the HindIII and Not I sites;

Figure 11 shows a schematic diagram of plasmid pSK491 as used in the present invention. The plasmid contains the chimeric regulator being a translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the PstI site at nucleotide 674 to the end of the coding region at nucleotide 839, amino acids 219 to 279 of the C1 protein) inserted into pBluescript between the HindIII and Not I sites.,

Figure 12 shows a schematic diagram of plasmid pUCAP (van Engelen *et al.*, 1995),

Figure 13 shows a schematic diagram of plasmid pDV35S2 as used in the present invention. The plasmid is the pUCAP plasmid with the CaMV 35S promoter/terminator fragment from pDV35S1;

Figure 14 shows a schematic diagram of plasmid pSK10489 as used in the present invention. The plasmid contains the ohpR sequence inserted into the BamHI and XbaI sites between the CaMV 35S promoter and the CaMV 35S terminator in pDV35S1;

Figure 15 shows a schematic diagram of plasmid pSK10490 as used in the present invention. The plasmid contains the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA from the NarI at nucleotide 536 to the end of the coding region at nucleotide 839, amino acids 179 to 279 of the C1 protein) from plasmid pSK490 inserted into the BamHI and XbaI sites between the CaMV 35S promoter and the CaMV 35S terminator in pDV35S1;

Figure 16 shows a schematic diagram of plasmid pSK10491 as used in the present invention. The plasmid contains the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the PstI site at nucleotide 674 to the end of the coding region at nucleotide 539, amino acids 219 to 279 of the C1 protein) from plasmid pSK491 inserted into the BamHI and XbaI sites between the CaMV 35S promoter and the CaMV 35S terminator in pDV35S1;

Figure 17 shows a schematic diagram of plasmid pBNP as used in the present invention. The plasmid is also known as pBINplus (van Engelen 1995);

Figure 18 shows a schematic diagram of plasmid pBNP58040 as used in the present invention. The plasmid contains the SmaI/SacI DNA fragment from pSK58040 inserted into the SmaI/SacI sites in pBINplus. This fragment contains the ohp operator from nucleotide 1036 to nucleotide 1449 of SEQ ID NO: 1 upstream of the CaMV 35S –90 bp core promoter, GUS intron (Vancanneyt *et al.*, 1990) and nos terminator;

Figure 19 shows a schematic diagram of plasmid pBNP60040 as used in the present invention. The plasmid contains the XhoI/SacI fragment from pSK60040 inserted into the XhoI/SacI sites in pBINplus. This fragment contains the chimeric promoter (Seq. ID. No: 13), a GUS intron (Vancanneyt *et al.*, 1990) and a nos terminator;

Figure 20 shows a schematic diagram of the plasmid pBNP10489 as used in the present invention. The plasmid contains the HindIII/SacI fragment from pSK10489 inserted into the HindIII/SacI sites in pBINplus. This fragment contains the ohpR sequence inserted between the CaMV 35S promoter and the CaMV 35S terminator;

Figure 21 shows a schematic diagram of the plasmid pBNP10490 as used in the present invention. The plasmid contains the HindIII/SacI fragment from pSK10490

inserted into the HindIII/SacI sites in pBINplus. This fragment contains the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the NarI at 536 bp to the end of the coding region at nucleotide 839, amino acids 179 to 279 of the C1 protein) inserted between the CaMV 35S promoter and the CaMV 35S terminator in pDV35S;

Figure 22 shows a schematic diagram of the plasmid pBNP10491 as used in the present invention. The plasmid contains the HindIII/SacI fragment from pSK10491 inserted into the HindIII/SacI sites in pBINplus, This fragment contains the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the PstI site at nucleotide 674 to the end of the coding region at nucleotide 839, amino acids 219 to 279 of the C1 protein) inserted between the CaMV 35S promoter and the CaMV 35S terminator in pDV35S;

Figure 23 shows a schematic diagram of the plasmid pOH001 as used in the present invention. The plasmid is a double construct in pBINplus containing the ohp operator from nucleotide 1036 to nucleotide 1449 of SEQ ID NO: 1 upstream of the CaMV 35S –90 bp core promoter, GUS intron and nos terminator, and also containing the ohpR sequence between the CaMV 35S promoter and the CaMV 35S terminator;

Figure 24 shows a schematic diagram of the plasmid pOH003 as used in the present invention. The plasmid is a double construct in pBINplus containing the ohp operator from nucleotide 1036 to nucleotide 1449 of SEQ ID NO: 1 inserted upstream of the CaMV 35S –90 bp core promoter, GUS intron and nos terminator, and also containing the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the NarI at nucleotide 536 to the end of the coding region at nucleotide 839, amino acids 179 to 279 of the C1 protein) between the CaMV 35S promoter and the CaMV 35S terminator;

Figure 25 shows a schematic diagram of the plasmid pOH004 as used in the present invention. The plasmid is a double construct in pBINplus, containing the ohp operator from nucleotide 1036 to nucleotide 1449 of SEQ ID NO: 1 upstream of the CaMV 35S –90 bp core promoter, GUS intron and nos terminator, and also containing the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the PstI site at nucleotide 674 to the end of the coding region at nucleotide 839, amino acids 219 to 279 of the C1 protein) between the CaMV 35S promoter and the CaMV 35S terminator;

Figure 26 shows a schematic diagram of the plasmid pOH005 as used in the present invention. The plasmid is a double construct in pBINplus, containing the chimeric promoter (Seq. ID. 13), a GUS intron (Vancanneyt *et al.*, 1990) and a nos terminator, and

also containing the ohpR sequence between the CaMV 35S promoter and the nos terminator and also containing the ohpR sequence between the CaMV 35S promoter and the CaMV 35S terminator;

Figure 27 shows a schematic diagram of the plasmid pOH006 as used in the present invention. The plasmid is a double construct in pBINplus, containing the chimaeric promoter (Seq. ID. 13), a GUS intron (Vancanneyt *et al.*, 1990) and a nos terminator, and also containing the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the NarI at nucleotide 536 to the end of the coding region at nucleotide 839, amino acids 179 to 279 of the C1 protein) between the CaMV 35S promoter and the CaMV 35S terminator;

Figure 28 shows a schematic diagram of the plasmid pOH007 as used in the present invention. The plasmid is a double construct in pBINplus containing the chimeric promoter (Seq. ID. 1), a GUS intron (Vancanneyt *et al.*, 1990) and a nos terminator, and also containing the translational fusion between the ohpR coding sequence (nucleotide 295 to nucleotide 1035 of SEQ ID NO: 1) and part of the C1 cDNA (from the PstI site at nucleotide 674 to the end of the coding region at nucleotide 839, amino acids 219 to 279 of the C1 protein) between the CaMV 35S promoter and the CaMV 35S terminator.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of controlling eukaryotic gene expression comprising introducing into or transforming a eukaryotic cell with (i) an inducible gene expression system, comprising a first nucleotide sequence comprising a first 5' regulatory region operably linked to a nucleic acid sequence which encodes a regulator polypeptide and an untranslated 3' termination sequence, and (ii) a second nucleotide sequence comprising a second 5' regulatory region operably linked to a nucleic acid sequence which is a coding or non-coding sequence (*i.e.*, the target gene or sequence), the expression of the nucleic acid sequence of the second nucleotide sequence being controlled by the regulator polypeptide of the first nucleotide sequence using an inducer. The inducer thereby causes modulation of expression of the nucleic acid sequence of the second nucleotide sequence (the target gene). The nucleotide sequence of the regulator polypeptide and/or the second 5' regulatory region, or parts thereof, of the second nucleotide sequence are preferably isolated from a prokaryote source.

While the first nucleotide sequence of the method and chimeric gene hereof advantageously comprise an untranslated 3' termination sequence, a termination sequence may not be essential to the operation of the inducible expression system.

Advantageously the inducible gene expression system is a chemically inducible gene expression system.

Preferably, one or more of the 5' regulatory regions each comprises a promoter which allows expression in eukaryote cells and/or tissues.

Appropriate promoters are chosen so that expression of the regulator polypeptide may be constitutive, developmentally regulated, tissue-specific, cell-specific or cell compartment-specific. Suitable constitutive promoters include but are not limited to CaMV 35S and CaMV 19S promoters.

Suitable tissue specific promoters include but are not limited to the patatin promoter and the *petE* promoter.

Suitable cell compartment promoters include but are not limited to promoters of chloroplast genes, such as the gene encoding the large subunit of ribulose biphosphate carboxylase and promoters of mitochondrial genes, such as the 18S-5S rRNA genes. Other suitable promoters will be known to one skilled in the art.

Advantageously, the 5' regulatory regions may also comprise one or more enhancer sequences. The enhancer sequence may be a transcriptional and/or translational enhancer sequence.

Numerous sequences have been found to enhance gene expression in transgenic plants. Suitable translational enhancer sequences include a number of non-translated leader sequences derived from viruses are known to enhance expression. Specifically, leader sequences from Tobacco Mosaic Virus (TMV), Maize Chlorotic Mottle Virus (MCMV) and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (e.g. Gallie *et al.*, 1987, Skuzeski *et al.*, 1990). Other leader sequences known in the art include but are not limited to: Picornavirus leaders, Potyvirus leaders, AMV RNA4 leader (Jobling & Gehrke 1987) or the HSP 70 leader (disclosed in US Patent No. 5,659,122).

Suitable transcriptional enhancer sequences will be known to those skilled in the art, such as the *petE* enhancer disclosed in our International Patent Application, Publication No. WO 97/20056.

Various intron sequences have been shown to enhance expression when added to the 5' regulatory region. For example, the introns of the maize Adh1 gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells (Callis *et al.*, 1987). International Patent Application, Publication No. WO 9319189 discloses the use of the Hsp70 intron from maize to enhance gene expression in transgenic plants.

Advantageously, the regulator polypeptide comprises one or more domains, which domains may be a ligand binding domain, a nucleic acid binding domain, a transactivation domain, a targeting domain, a silencing/repressing domain or a dimerization domain. The regulator sequence may thus comprise a chimeric gene of different sequences.

The ligand binding domain suitably comprises a sequence of amino acids whose structure binds non-covalently a complementary ligand. The ligand may be a chemical ligand. Hence, a ligand binding domain and its ligand form a complementary binding pair. Ligand binding domains for the construction of chimaeric regulator polypeptides may also be obtained from a variety of sources. The complementary ligand may be the inducer, a derivative or a precursor of the inducer.

It is possible to use two or more chemical ligands that may act together as synergists and/or antagonists. The source of chemical ligand will depend on which ligand binding domains are present in the regulator polypeptide. Any chemical compound will suffice as long as it is shown to form a complementary binding pair with the chosen ligand binding domain.

The nucleic acid binding domain comprises a sequence of amino acids which binds non-covalently to a specific nucleotide sequence known as a response element (RE). The response element may be located in the 5' regulatory region of the second nucleotide sequence. The nucleotide sequence and linear orientation determines which nucleic acid binding domain or domains will form a complementary binding pair with the response element. Considerable flexibility can be introduced into the method of controlling gene expression by using these conserved response elements in other ways.

Additional flexibility in controlling gene expression may be obtained by using nucleic acid binding domains and response elements from other nucleic acid binding proteins, which include but are not limited to the LexA, Gal4, LacI, Tet, C1 and Ace1 proteins described above.

A further degree of flexibility in controlling gene expression can be obtained by combining response elements which form complementary binding pairs with nucleic acid binding domains from different types of nucleic acid binding proteins, i.e. overlapping response elements.

The transactivation domain comprises one or more sequences of amino acids acting as subdomains which affect the operation of transcription factors during pre-initiation and assembly at the TATA box. The effect of the transactivation domain is to allow repeated transcription initiation events, leading to greater levels of gene expression. Different transactivation domains are known to have different degrees of effectiveness in their ability to increase transcription initiation. In the present invention, it is desirable to use

transactivation domains which have superior transactivating effectiveness in eukaryotic cells in order to create a high level of target gene expression in eukaryotic cells. Transactivation domains which have been shown to be particularly effective include but are not limited to Vp16 (isolated from the herpes simplex virus) and C1 isolated from maize. Other transactivation domains known to those skilled in the art will also be effective.

The silencing/repressing domain comprises one or more sequences of amino acids acting as subdomains which affect the RNA polymerase II basal or regulatory transcription machinery. The effect of the silencing/repressing domain is to stop the progression of transcription. Different silencing/repressing domains are known to have different degrees of effectiveness in their ability to decrease transcription. In the present invention, it is desirable to use silencing/repressing domains which have superior silencing/repressing effectiveness in eukaryotic cells in order to create a high level of target gene repression in eukaryotic cells. Silencing/repression domains which have been shown to be particularly effective include but are not limited to the KRAB domains identified in human, mouse and *Xenopus* zinc finger proteins (for review see Hanna-Rose & Hansen 1996) and the Oshox1 protein of rice (Meijer *et al.*, 1997). Other silencing/repressing domains known to those skilled in the art will also be effective.

The dimerization domain comprises one or more sequences of amino acids acting as subdomains which affect the protein-protein interaction. Different dimerization domains are known to have different degrees of effectiveness in their ability to form protein-protein interactions. In the present invention, it is desirable to use dimerization domains which have superior dimerization effectiveness in eukaryotic cells in order to create a high level of protein-protein interaction in eukaryotic cells. Dimerization domains which have been shown to be particularly effective include but are not limited to Helix-loop-helix domains of Myc and MycoD and the leucine zipper domains of Myc and GCN4 proteins. Other dimerization domains known to those skilled in the art will also be effective.

The targeting domain may comprise targeting polypeptides to direct the regulator sequence to different parts of eukaryotic cells. Suitable targeting domains include but are not limited to examples such as a plasma membrane targeting sequence (Hedley *et al.*, 1993), golgi , endoplasmatic reticulum (Iturriaga *et al.*, 1989), nuclear targeting signals (Varagona *et al.*, 1992, Raikhel 1992), chloroplast (Rensink *et al.*, 1998), mitochondrial (Boutry *et al.*, 1987) or inner envelope targeting sequences (Knight & Gray 1995).

The nucleotide sequences which encode any of the above domains may advantageously be modified for improved expression in eukaryotes, have altered functionality, or both. Such modifications include, but are not limited to, altering codon

usage, insertion of introns or creation of mutations, preferably in the ligand binding domain and/or the nucleotide binding domain. Modified nucleotide sequences of the regulatory sequence are an aspect of the present invention.

Furthermore, ligand-binding, nucleic acid binding, transactivation and targeting domains may be assembled in a chimeric regulator polypeptide in any functional arrangement.

Chimeric regulator polypeptides may also have multiple domains of the same type, for example, more than one transactivation domain or nucleic acid binding domain per regulator polypeptide. Mutant regulator polypeptides may be prepared by methods of mutagenesis known in the art, such as chemical mutagenesis or site-directed mutagenesis. This might result in ligand binding domains with altered ligand binding and/or nucleic acid binding domains with altered recognition sites.

Advantageously the regulatory sequence comprises a ligand binding domain and/or a DNA binding domain.

Preferably, the regulator sequence is the nucleotide sequence from 295-1035bp of SEQ ID NO: 1. Advantageously the sequence may be isolated from the ohpR sequence in *Rhodococcus* sp. V49. Subsequences of this sequence having the necessary function may also be used in the invention.

Rhodococcus sp. V49 encodes the OHP catabolic operon, which is presented in SEQ ID NO: 1, which sequence shows the nucleotide sequences among others of the ohpR, the ohpA operator region (1036-1260bp), ohpA, OhpB, OhpC and OhpD genes, which when expressed allow growth on OHP as sole carbon-energy source. SEQ. ID. Nos. 2 through 7 represent amino acid sequences of the proteins encoded by the OHP catabolic operon, for example, ohpR regulator (SEQ ID NO: 2), ohpA transport (SEQ ID NO: 3), OhpB monoxygenase (SEQ ID NO: 4), OhpD catechol 2,3-dioxygenase (SEQ ID NO: 5), and OhpC hydrolase (SEQ ID NO: 6). Nucleic acid sequences substantially similar to those sequences or nucleic acid sequences encoding proteins with similar functionality may also be suitable for aspects of the present invention.

Gene sequence similarity is established by Southern Blot screening. Such screening is initially carried out under low-stringency conditions, which comprise a temperature of about 37°C or less, a formamide concentration of less than about 50%, and a moderate to low salt (e.g. Standard Saline Citrate ('SSC') = 0.15 M sodium chloride; 0.15 M sodium citrate; pH7) concentration. Alternatively, a temperature of about 50°C or less and a high salt (e.g. SSPE= 0.280 mM sodium chloride; 9 mM disodium hydrogen phosphate; 9 mM sodium dihydrogen phosphate; 1 mM sodium EDTA; pH 7.4). Preferably the screening is carried out at about 37°C, a formamide concentration of about 20%, and a

salt concentration of about 5 x SSPE. These conditions will allow the identification of sequences which have a substantial degree of similarity with the probe sequence, without requiring the perfect homology for the identification of a stable hybrid. The phrase 'substantial similarity' refers to sequences which share at least 50% overall sequence identity. Preferably, hybridisation conditions will be selected which allow the identification of sequences having at least ~~70%~~ sequence identity with the probe, while discriminating against sequences which have a lower level of sequence identity with respect to the probe. After low stringency hybridisation has been used to identify several bacterial whose genome or DNA sub-clones exhibit a substantial degree of similarity with the probe sequence, this subset of genomes or sub-clones is then subjected to higher stringency hybridisation, so as to identify those of this subset of genomes or sub-clones having a particularly high level of homology with respect to the probe sequences. Medium stringency conditions comprise a temperature of about 39°C and a medium salt (SSC) concentration. High stringency conditions comprise a temperature of about 42°C or less, and a low salt (SSC) concentration. Alternatively, they may comprise a temperature of 65°C or less, and a low salt (SSPE) concentration. Preferred conditions for such screening comprise a temperature of about 42°C, a formamide concentration of about 20%, and a salt concentration of about 2xSSC, or a temperature of about 65°C, and a salt concentration of about 0.2 SSPE.

Suitable untranslated 3'termination sequences such as the CaMV 35S or *nos* terminator will be known to those skilled in the art.

Preferably, the 5' regulatory region of the second nucleotide sequence may also comprise a core promoter sequence and the response element (RE) or response elements necessary for complementary binding of the regulator polypeptide. By core promoter it is intended that the basal promoter elements are inactive or nearly so without activation. Such a promoter has low background activity in eukaryotes when there is no transactivator present, or when enhancer or response element binding sites are absent. Core promoters that are particularly useful for target genes in plants are the A1 core promoter which is obtained from the A1 gene of maize (Tuerck & Fromm, 1994) or the CaMV35s core promoter.

Alternatively, the 5' regulatory region of the second nucleotide sequence may also comprise a full-length promoter sequence and the response element (RE) or response elements necessary for complementary binding of the regulator polypeptide. Such a promoter has high activity in eukaryotes when there is no transactivator present. Full-length promoters that are particularly useful for target genes in plants are the CaMV 35S promoter, the CERV promoter and the petE promoter.

Preferably, the response element of the 5' regulatory region of the second nucleotide sequence is derived from the nucleotide sequence seen from nucleotide 295 to nucleotide 2805 in SEQ ID NO: 1. Advantageously the sequence is isolated from the ohpA promoter region (nucleotides 1036-1260 of SEQ ID NO: 1) in Rhodococcus sp. V49 (ATCC19070). Subsequences of this sequence having the necessary function and/or multiples of this sequence or subsequences can be used in the present invention in normal or reverse orientation, upstream or downstream of the core promoter, and in any order thereof. Substantially similar sequences to the ohpR-ohpA region in accordance with the hybridization conditions described above are also within the scope of the present invention.

Suitable coding sequences in the second nucleotide sequence include, but are not limited to, sequences which encode proteins involved in carbon metabolism; flowering; fertility and/or sterility, for example, the use of barnase or diptheria toxin A-chain; cell wall metabolism; genes that respond to environmental signals, for example pathogen attack, such as nematode, arachnid or aphid attack; or bacterium, fungus, virus, or insect resistance; or genes that confer resistance to antibiotics, herbicides or other toxic compounds.

The coding sequence may be homologous or heterologous in origin with respect to the eukaryote being transformed.

Sense, co-suppression or anti-sense technology may be used as required to achieve alteration of the eukaryote.

Nucleotide sequences may be introduced into the cell by any method known to one skilled in the art. Transformation techniques such as the use of *Agrobacterium*, microinjection, microprojectile-bombardment, electroporation and others known to the skilled man are among those methods for which this invention is appropriate.

The expression of the nucleic acid sequence of the second nucleotide sequence (also known herein as the target gene) may be suitably increased or decreased, whether from a basal or medial level respectively, or completely repressed or activated.

Advantageously, an increase in target gene expression levels may be caused by the addition or presence of the inducer. Alternatively, an increase in target gene expression levels may be caused by the withdrawal or absence of the inducer. Similarly, a decrease in target gene expression levels may be caused by the addition or presence of the inducer, or alternatively, a decrease in target gene expression levels may be caused by the withdrawal or absence of the inducer.

Preferably, the inducer which causes modulation of expression of the nucleic acid sequence is a chemical compound, such as OHP, 2-hydroxy cinnamic acid, toluene, bezene, n-hexadecane or a functional equivalent of either. The inducer may also, however, be a protein or nucleic acid sequence, depending on the complementary domain of the

regulator sequence. The 5' regulatory region of the second nucleotide sequence may suitably comprise one or more response elements, each being necessary for complementary binding of an appropriate domain or other portion of the regulator sequence.

Advantageously the inducer acts by indirect action. Alternatively, the inducer acts by direct action.

Preferably the eukaryotic cell is a plant cell. The plant cell may be one or more from the group consisting of, for example, crops such as potato, wheat, maize, barley, tomato, rice, canola, sugarbeet or tobacco; trees such as eucalyptus species, populus or malus; or other plants, such as *Arabidopsis*.

Preferably the gene expression system comprises a single construct containing the first nucleotide sequence and the second nucleotide sequence. In the alternative, the gene expression system may utilise two or more separate constructs, and further each construct may be introduced into separate eukaryotes, which are then transferred into one eukaryote, biologically mated or crossed, for example, to bring the constructs together.

Alternatively, the expression system may comprise one transformation step followed by a further transformation step or steps. Each step may introduce one or more additional constructs, for example, co-transformation or re-transformation.

The present invention also provides a chimeric gene comprising a first nucleotide sequence comprising a first 5' regulatory region operably linked to a nucleic acid sequence which encodes a regulator polypeptide and an untranslated 3' termination sequence, and a second nucleotide sequence comprising a second5' regulatory region operably linked to a nucleic acid sequence which is a coding or non-coding sequence (*i.e.*, target gene or sequence), the expression of the nucleic acid sequence of the second nucleotide sequence being controlled by the regulator polypeptide of the first nucleotide sequence using an inducer. The inducer thereby causes modulation of expression of the nucleic acid sequence of the second nucleotide sequence (the target gene). The nucleotide sequence of the regulator polypeptide and/or the second 5' regulatory region or parts thereof of the second nucleotide sequence are isolated preferably from a prokaryote source.

Alternatively there may be provided a first chimaeric gene comprising the first nucleotide sequence and a second chimaeric gene comprising the second nucleotide sequence.

Advantageously the chimaeric gene is utilised in a plasmid, vector or other transportable medium suitable for microbiological genetic transformation.

Plant tissue, such as cells, organs, seed and other plant parts transformed using the aspects of the present invention are also aspects of the instant invention.

6. EXAMPLES

In order to transform eukaryotes the preparation of constructs and the use of transformation techniques are required in accordance with the following Examples.

6.1 MATERIALS AND METHODS

Generally speaking, those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression in common hosts such as *E. coli* and *Agrobacterium*. Suitable vectors for the construction of gene expression cassettes can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details, see, for example, Molecular Cloning: A Laboratory manual: 2nd edition, Sambrook *et al.* 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing and, introduction of DNA into cells, gene expression, and analysis of proteins are described in detail in Current Protocols in Molecular Biology, Second Edition, Ausubel *et al.*, eds, John Wiley and Sons 1992. The disclosures of Sambrook *et al.*, and Ausubel *et* al., are incorporated herein by reference.

However the present inventors have recognized that certain methods previously employed in the art which were developed for enteric bacteria such as E. coli may not be most appropriate for use in plant genetic constructs. Accordingly, advantageous methods have been developed by the inventors which in preferred forms allow the rapid construction of OHP genetic constructs and operably linked inducible 5' regulatory regions and regulator constructs.

The following examples further describe the materials and methods used in carrying out the invention and the subsequent results. They are offered by way of illustration, and their recitation should not be considered as a limitation of the claimed invention.

6.2 Isolation of the ohpR sequence

Example 1

The coding sequence of the OHP operon OhpR (from nucleotide 295 to nucleotide 1035) was amplified by PCR from construct pJP58 using the primers OHPR3 (SEQ ID NO: 8) and OHPR4 (SEQ ID NO: 9). The construct pJP58 was deposited by Advanced Technologies (Cambridge) Ltd of 210 Cambridge Science Park, Cambridge CB4

0WA, England under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the purposes of Patent Procedure at the National Collection of Industrial and Marine Bacteria (NCIMB), 23 St. Machar Street, Aberdeen, Scotland on 21st December 1998 under accession number NCIMB 40997. It contains a 2 kb BamHI fragment encoding the ohpA-ohpR region (nucleotides 1-1869 of SEQ ID NO: 1) cloned into pUC19 using the unique BamHI site (Veira J. & Messing, J. 1982).

The PCR product was restriction digested with EcoRI and NotI and cloned into pBluescript (Stratagene) also digested with EcoRI and NotI. The resulting plasmid was named pSK489 (Figure 1) and sequenced.

6.3 **Isolation of the transcriptional activator sequence C1**

Example 2

The C1 cDNA region was amplified by PCR from plasmid p35SC1 (Figure 2), as described in Tuerck & Fromm (1994), using the primers C11 (SEQ ID NO: 10) and C12 (SEQ ID NO: 11). The PCR product was digested with EcoRI and NotI and ligated into pBluescript digested with EcoRI and NotI. The resulting plasmid was named pSK483 (Figure 3) and sequenced.

6.4 **Isolation of the operator sequence**

Example 3

Part of the operator region of the OHP operon (from nucleotide 1036 to nucleotide 1449 in SEQ ID NO: 1) was amplified by PCR from construct pJP58 using the oligonucleotide primers op1 (SEQ ID NO: 12) and op2 (SEQ ID NO: 13). The 441bp PCR product was restriction digested to with Xho1 and Sal1, gel-purified and ligated into pBluescript digested with XhoI and SalI. The resulting plasmid was named pSK-59 (Figure 4) and sequenced.

6.5 **Construction of the construct for the nucleic acid sequence in the second nucleotide sequence**

Example 4

The plasmid pSK-59 (Figure 4) was digested with Xho1 and Sal1, the 414bp operator region was gel-purified and ligated with pBS52040 (Figure 5) which had been digested with XhoI and phosphatased. The resulting plasmid was named pSK58040 (Figure 6).

6.6 Construction of a chimeric CaMV35S promoter-ohp regulator construct

Example 5

The three oligonucleotides CaMVop2 (SEQ ID NO: 14), CaMVop3 (SEQ ID NO: 15) and CaMVop4 (SEQ ID NO:16) were annealed in equimolar amounts (500 pmole each primer) and diluted tenfold. 5 µl of this dilution were used as a template for a PCR reaction (50µl total) catalysed by a proof-reading Taq polymerase to generate double stranded product. The PCR product was resolved on an 8% polyacrylamide gel. The 125 bp PCR product was excised and purified using techniques described in Sambrook et al (1989). 1 µl of the total eluted double stranded DNA solution (50µl) was used as a template in a PCR reaction (50µl total) primed by oligonucleotide primers CaMVopF1 (SEQ ID NO: 17) and CaMVopR1 (SEQ ID NO: 18) and catalysed by a proof-reading Taq polymerase. The PCR product from this reaction was digested to completion with EcoRV and BamHI and the 133 bp restriction fragment ligated with plasmid pDV35S1 (Figure 7) similarly digested to completion with EcoRV and .the resulting construct was named pDV60 (Figure 8). The inserted region was sequenced. Plasmid pDV60(Figure 8) was digested with XhoI and BamHI. The 476bp synthetic promoter restriction fragment (SEQ ID NO: 19) was gel purified as described above and ligated into pSK52040 (Figure 5) similarly digested with XhoI and BamHI. This plasmid was named pSK60040 (Figure 9). The chimeric promoter in SEQ ID NO: 18 contains a 36 bp region of the ohp operon (from nucleotide 1225 to nucleotide 1260) inserted into the CaMV 35S promoter at nucleotide 21.

6.7 Construction of chimeric regulator sequences

Example 6

The plasmid pSK483 (Figure 3) was digested to completion with PstI and XbaI. The 162bp fragment (the C1 cDNA region from amino acids 219 to 273 of the C1 protein) was gel-purified and ligated with pSK489 (Figure 1) similarly digested with PstI and XbaI. The resulting plasmid was named pSK491 (Figure 11). This ligation results in a translation fusion of the OHPR nucleotide sequence and the C1 nucleotide sequence for the transcriptional activation domain from amino acid 219 to 273.

The plasmid pSK483 (Figure 3) was also digested to completion with NarI and XbaI. The 303bp fragment of the C1 cDNA region (encoding amino acids 173 to 273 of the C1 protein) was gel-purified and ligated with pSK489 (Figure 1) similarly digested with NarI and XbaI. The resulting plasmid was named pSK490 (Figure 10). This ligation results in a translation fusion of the ohpR nucleotide sequence and the C1 nucleotide sequence for the transcriptional activation domain from amino acid 173 to 273.

6.8 Construction of pDV35S2

Example 7

pDV35S1 (Figure 7) was digested with HindIII and SacI and the 668 bp fragment containing the CaMV 35S promoter/terminator was gel-purified and ligated with pUCAP (Figure 12) which was digested with HindIII and SacI. The resulting construct was named pDV35S2 (Figure 13).

6.9 Construction of a regulator expression construct

Example 8

Plasmids pSK489 (Figure 1), pSK490 (Figure 10) and pSK491 (Figure 11) were digested with BamHI and XbaI, the fragments encoding the regulator sequences were gel-purified and ligated with pDV35S1 (Figure 7), similarly digested with BamHI and XbaI. The resulting plasmids were named pSK10489 (ohpR, Figure 14), pSK10490 (ohpR-C1 NarI/XbaI fusion, Figure 15) and pSK10491 (ohpR-C1 PstI/XbaI fusion, Figure 16) respectively.

6.10 Construction of pBNP58040

Example 9

Plasmid pSK58040 (Figure 6) was digested to completion with HindIII and SmaI and the 2837 bp fragment containing the CaMV 35S promoter-GUS-nos terminator was gel-purified and ligated into pBINplus (Figure 17) similarly digested with HindIII and SmaI. The resulting plasmid was named pBNP58040 (Figure 18).

6.11 Construction of pBNP60040

Example 10

Plasmid pSK60040 (Figure 9) was digested to completion with HindIII and SacI and the promoter-Gus fragment was gel-purified and ligated with pBINplus (Figure 17) similarly digested with HindIII and SacI. The resulting plasmid was named pBNP60040 (Figure 19).

6.12 Construction of plant transformation vectors carrying the regulat r genes.

Example 11

The regulator cassettes were cut out of pSK10489 (Figure 14), pSK10490 (Figure 15), and pSK10491 (Figure 16), respectively. DNA was digested with HindIII and SacI. The restriction fragments containing the CaMV 35S promoter-regulator were gel-purified. The isolated fragments were ligated with pBINplus (Figure 17) similarly digested with HindIII and SacI. The resulting plasmids were named pBNP10489 (Figure 20 - pBNP containing 10489, ohpR), pBNP10490 (Figure 21 - pBNP containing 10490, ohpR-C1 NarI fusion) and pBNP10491 (Figure 22 - pBNP containing 10491, ohpR-C1 PstI fusion).

6.13 Construction of double gene expression constructs

Example 12

The CaMV 35S promoter-regulator fragments were cut out of pSK10489 (Figure 14), pSK10490 (Figure 15) and pSK10491 (Figure 16) respectively. DNA was digested with NotI, blunt-ended with Klenow DNA polymerase and then digested with HindIII. The restriction fragments containing the CaMV 35S promoter/regulator were gel-purified. pBNP58040 (Figure 18) and pBNP60040 (Figure 19) were digested with HindIII and SmaI. The gel-purified fragments were ligated with either the digested pBNP58040 (Figure 18) or the digested pBNP60040 (Figure 19). The resulting plasmids were named pOH001 (Figure 23 - pBNP containing 58040 and 10489), pOH003 (Figure 24 - pBNP containing 58040 and 10490), pOH004 (Figure 25 - pBNP containing 58040 and 10491), pOH005 (Figure 26 - pBNP containing 60040 and 10489), pOH006 (Figure 27 - pBNP containing 60040 and 10490) and pOH007 (Figure 28 - pBNP containing 60040 and 10491).

6.14 Transformation of *Agrobacterium*

Example 13

The plant transformation vectors (as described in Examples 9-12, Figures, 18-28) were electroporated into *Agrobacterium tumefaciens* cells. Agrobacterium cultures were selected on kanamycin-containing medium (50µg/ml). The cultures were grown in liquid medium and then used for the transformation of plant species.

6.15 Transformation or Retransformation of plants

Example 14

Tobacco and potato plants can be transformed using the method of leaf disk cocultivation as essentially described by Horsch *et al.*, (1985). The binary vectors as described above in Examples 9-12 (Figures 18-28) are transferred to *Agrobacterium tumefaciens* LBA4404 using the method of electroporation, and cultures of said *Agrobacteria* can be used in transformation so that regenerated plants carry the chimeric genes as described in Examples 9-12.

Young leaves were dissected under sterile conditions, from approximately 4 week old *Eucalyptus* species cultures grown in Magenta boxes (7 cm x 7 cm x 13 cm) on LS media at 25 C, in a growth room in our tissue culture laboratory and used for *Agrobacterium*-mediated transformation (Horsch et al. 1985) using the strain EHA105. Inoculated tissue was left to co-cultivate for 4 days on LS media (plus 20-g/l glucose, 0.7 % agarose, 0.1 mM Zeatin and 1μM NAA) in diffuse light in a growth, conditions as before. Transformants were selected on 50 mg/ml kanamycin and 250 mg/ml claforan.

Arabidopsis thaliana was transformed following the protocols from Bechthold *et al.*, (1993) and Clough (1998). Plants were grown in a growth cabinet at 22°C under 18h daylight before and after vacuum-infiltration.

Several direct gene transfer procedures have been developed to transform plants and plant tissues without the use of an *Agrobacterium* intermediate. In the direct transformation of protoplasts the uptake of exogenous genetic material into a protoplast may be enhanced by use of a chemical agent or electric field. The exogenous material may then be integrated into the nuclear genome (Pazkowski *et al.*, 1984, Potrykus *et al.*, 1985). Alternatively, exogenous DNA can be introduced into cells or protoplasts by microinjection. A solution of plasmid DNA is injected directly into the cell with a finely pulled glass needle (Reich *et al.*, 1986). A more recently developed procedure for direct gene transfer involves bombardment of cells by microprojectiles carrying DNA (Klein *et al.*, 1987). In this procedure tungsten or gold particles coated with the exogenous DNA are accelerated towards the target cells, resulting in transient expression and also in stable integration of the DNA into the plant genome.

Following transformation, the transformed cell or plant tissue is selected or screened by conventional techniques. The transformed cell or plant tissue contains the chimeric DNA sequences discussed above and is the regenerated, if desired, by known procedures. The regenerated plants are screened for transformation by standard methods. Progeny of the regenerated plants is continuously screened and selected for the continued presence of the integrated DNA sequence in order to develop improved plant and seed lines.

The DNA sequence can be moved into other genetic lines by a variety of techniques, including classical breeding, protoplast fusion, nuclear transfer and chromosome transfer.

The chimeric binary vector plasmids mentioned above can be used to transform a plant already carrying other chimeric genes by the methods described above.

6.16 Transient expression

Example 15

Transient expression assays of the gene expression cassette was essentially performed as described by Kapila *et al.*, (1997), Rossi *et al.*, (1993), Twell *et al.*, (1989), Goff *et al.*, (1990), Roth *et al.*, (1991) and Tuerck *et al.*, (1994).

Leaf discs of 4-6 weeks old plants were excised and incubated with the *Agrobacterium* suspension. The discs were incubated for 1-5 days on wet Whatman paper before they were stained for GUS expression before and after induction.

6.17 Induction of reporter gene activity in transgenic plants

Example 16

OHP was applied to the plants (or plant cells) as a paint, spray or in the medium in concentrations ranging from 0.01 mM to 100 mM in water or in 10 mM MES, pH5.6. Tissue was harvested prior to inducer application and at appropriate times after the application. The sample tissue was ground in extraction buffer and assayed for GUS reporter gene activity as described by Jefferson (1987). Tissue was also stained for GUS expression as described by Jefferson (1987).

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

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